

# Effect of cold storage on shear-induced platelet aggregation and clot strength

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**BACKGROUND:** Platelets (PLTs) participate in hemostasis and save lives following trauma. PLTs for transfusion are maintained at room temperature (RT, 22°C), limiting viability to 5 days because of metabolic compromise and high risk of bacterial contamination. RT storage may result in weaker clots, delaying hemorrhage control, whereas cold storage (4°C) could permit longer PLT shelf life and result in a more hemostatic product. In this study, we characterized the effect of storage temperature on shear-induced PLT aggregation, clot formation, and strength.

**METHODS:** PLTs obtained from phlebotomized blood or by apheresis were stored at RT or 4°C for 5 days, and PLT aggregation and clot strength were assessed at 37°C. We studied PLT aggregation at steady and complex patterns of shear rates (500–2,500 per second) by flow cytometry, and the kinetics of clot formation and strength were measured using turbidity and dynamic mechanical analysis, respectively.

**RESULTS:** PLT aggregation was higher in 4°C-stored samples on Day 5 compared with fresh or RT-stored samples at all shear rates tested (fresh vs. 4°C and RT vs. 4°C,  $p < 0.05$ ). PLTs stored at 4°C for 5 days formed significantly stronger clots compared with fresh or RT-stored samples as quantified by turbidity and elastic moduli measurements (fresh vs. 4°C and RT vs. 4°C,  $p < 0.05$ ).

**CONCLUSION:** Our results show that cold-stored PLTs are more responsive to aggregation stimuli and form stronger clots, presumably because of thicker fibrin strands. These data suggest that the superior functionality of cold-stored PLTs may support faster hemostasis for acutely bleeding in trauma patients compared with RT-stored PLTs. (*J Trauma Acute Care Surg.* 2014;77: S88–S93. Copyright © 2014 by Lippincott Williams & Wilkins)

**KEY WORDS:** Platelet storage; refrigeration; SIPA; clot strength; hemorrhage.

Platelets are indispensable to hemostasis and are lifesaving when transfused to treat thrombocytopenia. In response to vascular injury, platelets adhere, aggregate, and along with fibrin, form a plug to mitigate blood loss. Every year, 9 million platelet products are used in the United States for various therapeutic and prophylactic indications.<sup>1</sup> Currently, platelets are stored at room temperature (RT) with gentle agitation for no more than 5 days to maximize recovery and survival in vivo after transfusion.<sup>2,3</sup> While RT storage decreases the need for frequent platelet transfusions in the setting of hematologic malignancies, it comes at the cost of efficacy and safety.<sup>4–9</sup> RT platelets are more metabolically active compared with refrigerated (4°C) platelets and accumulate functional defects over time, a phenomenon called the *platelet storage lesion*. Furthermore, 22°C-stored platelets have a higher risk of bacterial contamination, causing the US Food and Drug Administration to require bacterial testing and to limit their use to 5 days, after which they are discarded.<sup>8,10</sup>

Massive transfusion practices are changing to incorporate a higher ratio of platelets to red blood cells. Since platelets have no reliable substitute and the current donor pool is in short supply, the resulting increased demand can only be met by augmenting platelet inventories. Possible solutions include altering storage techniques to prolong shelf life. While cryopreserved platelets and similar products are under active investigation, refrigerated platelets are already a licensable product according to the US Food and Drug Administration Code of Federal Regulations (CFR) sections 21CFR640.24 and 21CFR640.25.<sup>4</sup> They retain better function according to previous work by our laboratory and others and, like all refrigerated blood products, are at lower risk of bacterial contamination.<sup>7,8,11</sup> Platelets were stored at 4°C from the 1960s until the 1980s, providing over a decade of safety data, but were gradually abandoned because of decreased survival in vivo.<sup>12</sup> While the decreased capacity of RT-stored platelets to halt active hemorrhage was discussed, the ultimate consensus in the transfusion medicine community was to maintain a single inventory stored at RT, primarily to serve patients requiring prophylactic transfusion.<sup>2,3,12</sup> This was done with the assumption that RT-stored PLTs would also benefit those with active hemorrhage, but the limited clinical data available suggest otherwise.

Two randomized clinical trials demonstrated that platelets stored at 4°C for 72 hours, either as whole blood or platelet concentrates, shortened the bleeding time and establish hemostasis in patients better than platelets stored at 22°C.<sup>13,14</sup> Several in vitro studies that compared RT-stored platelets with cold reported improved aggregation response, clot properties,

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hemostatic function, and metabolic reserve.<sup>5,7,11,15,16</sup> In light of these and other similar studies, reexamination of platelet refrigeration is currently underway at several centers to determine whether decreased accumulation of deficits and preservation of immediate hemostatic function are associated with better outcome after traumatic hemorrhage compared with longer in vivo circulation times.<sup>6,17,18</sup>

Two key measures of platelet component preservation are aggregation and clot stiffness. These properties have not been tested in refrigerated platelets at current storage limits under flow conditions, since shear stress caused by blood flow alters platelet activation and aggregation.<sup>19</sup> We hypothesized that 4°C platelet storage preserves clot stiffness and aggregation response to physiologic stimuli that mimic shear stresses experienced in circulation, better than platelets stored at 22°C.

## PATIENTS AND METHODS

### Ethics Statement

Venous blood was drawn from healthy volunteers after signing an informed consent and obtaining written regulatory approval in accordance with the institutional review board protocol (IRB #12-227, Office of Research Integrity and Compliance, University of Texas at San Antonio). Similarly, apheresis platelet (AP) units were collected from consented, healthy donors under a protocol reviewed and approved by the US Army Medical Research and Materiel Command Institutional Review Board and in accordance with the approved protocol.

### Platelet Storage and Handling

Platelet-rich plasma (PRP) was obtained from phlebotomized blood or AP collection. Blood was drawn in acid citrate dextrose-containing vacutainer tubes (BD Biosciences, San Jose, CA) and was spun at 250 G for 20 minutes (Eppendorf centrifuge 5430R, Hamburg, Germany) to obtain PRP. PRP was stored as 1-mL aliquots in 15-mL polypropylene centrifuge tubes with gentle agitation either at RT (20–24°C) or at 4°C (1–6°C). Single AP units were collected in acid citrate dextrose–plasma using a Trima Accel Automated Blood Collection System (Terumo BCT, Lakewood, CO), and 10-mL aliquots were aseptically transferred from the donor bags into 15-mL minibags (BCSI, Seattle, WA). The bags were stored with gentle agitation at RT or without agitation at 4°C. Platelet-poor plasma was obtained by centrifuging PRP or AP at 3,000 G for 20 minutes and collecting the supernatant. All assays were performed at 37°C.

### Shear-Induced Platelet Aggregation Assay

Platelets were exposed to shear stress in a cone-and-plate rheometer, and the aggregation was measured by flow cytometry.<sup>7</sup> Platelet concentration in fresh or stored samples was determined using Coulter counter (BD Biosciences). For shear-induced platelet aggregation (SIPA) experiments, platelet concentration was adjusted to  $3 \times 10^8$ /mL by diluting AP with autologous platelet-poor plasma. The platelet suspension was recalcified with  $\text{CaCl}_2$  at 20-mM final concentration. An 80- $\mu\text{L}$  aliquot of platelet suspension was placed on the plate of a cone-and-plate rheometer (MCR 302, Anton Paar, Germany)

and sheared at 37°C using a 25-mm, 1-degree cone at either constant (500 per second or 2,500 per second for 3 minutes) or complex-pattern (500 per second, 2,500 per second, and then 500 per second sequentially for 1 minute) shear rates to simulate the movement of platelets through complex vessel geometry. A 10- $\mu\text{L}$  aliquot of sheared sample was immediately fixed with 4% formaldehyde (Sigma, St. Louis, MO) and analyzed with a flow cytometer (FACSCalibur, BD Biosciences). Platelets were identified by their characteristic FSC/SSC pattern, and platelet aggregation was quantified as the percentage of that population located beyond the single platelet gate.

### Clot Turbidity Analysis

Clot kinetics was monitored by measuring turbidity using a spectrophotometer.<sup>20,21</sup> Clotting was initiated in fresh or stored PRP using 0.16-U human  $\alpha$ -thrombin (Haematologic Technologies Inc., Essex Junction, VT) and 3-mM calcium chloride to overcome citrate inhibition and activate factor XIII, promoting fibrin cross-linking. Reactions were measured in UV-transparent flat bottom 96-well plates (Corning Inc., Corning, NY) using a plate reader (BioTek, Winooski, VT) for 20 minutes at 350-nm UV absorbance and 37°C. PRP without thrombin and/or calcium was used as control. The rate of gelation was estimated from the initial slope of the absorbance curve and maximum turbidity from absorbance at steady state.

### Rheology

Cone-and-plate rheometry was used to quantify the changes in clot strength due to storage temperature.<sup>22</sup> During dynamic mechanical analysis, a steady constant strain of 0.5% is applied to a sample and the shear stress response was recorded until the response reaches steady state. At low strain (<1%), clots are in their linear viscoelastic regime, and small amplitude oscillations are used to track the evolution of elastic ( $G'$ ) and viscous moduli ( $G''$ ) through the gelation process. In these experiments, fresh or stored PRP (75  $\mu\text{L}$ ) was mixed with 0.16-U human  $\alpha$ -thrombin and 3-mM calcium chloride, then placed on a rheometer plate maintained at 37°C. An immiscible oil layer (Vapor-Lock liquid vapor barrier, Qiagen, Valencia, CA) was applied along the rim of the exposed surface to prevent evaporation, and the cone-and-plate setup was covered with a humidified chamber. Gelation kinetics was monitored for 30 minutes at 37°C at a constant small amplitude strain of 0.5% and a frequency of 1 Hz. The elastic and viscous moduli were estimated using the software supplied by the manufacturer (Rheoplus, Anton Paar, Germany).

### Statistical Analysis

The experiments were performed on platelets obtained from three to four different donors, and each experiment was performed at least in triplicate. The data were analyzed by one-way analysis of variance for repeated measures with a post hoc Tukey test and considered significant if  $p < 0.05$ . Graphical data are presented as mean  $\pm$  SEM. Microsoft Excel (Microsoft Corp., Redmond, WA) was used to manage data, and analysis was performed using GraphPad Prism software version 5 (GraphPad Prism Inc., San Diego, CA).

## RESULTS

### SIPA

Platelet microaggregate formation was quantified by SIPA assay as the increase platelet population size (Fig. 1A). We observed that shear stress had differential effects on fresh, RT-stored and 4°C-stored platelets (Fig. 1B), in that the fresh platelet response was modest but, as expected, aggregation increased with increased shear rate. RT-stored platelet aggregation was comparable with that of fresh platelets at all shear rates. In contrast, refrigerated platelets responded with aggregation levels that were fivefold higher compared with fresh platelets at steady and dynamic shear rates.

### Clot Turbidity Analysis

The time to clot formation, assessed via turbidity measurements, was similar between storage conditions (Fig. 2A). The rate of gelation, defined by the log phase of rapid fibrin polymerization, was significantly lower in RT-stored samples compared with fresh and 4°C-stored samples (Fig. 2B). Gel turbidity, a measure of fibrin density, was also influenced by storage conditions. Fresh and 4°C-stored samples formed more turbid or opaque gels compared with those at RT, a difference that could be seen visually (Fig. 2C). In combination, the qualitative and visual turbidity data suggest that 4°C storage results in better fibrin formation and stronger clot compared with RT storage.

### Clot Rheology

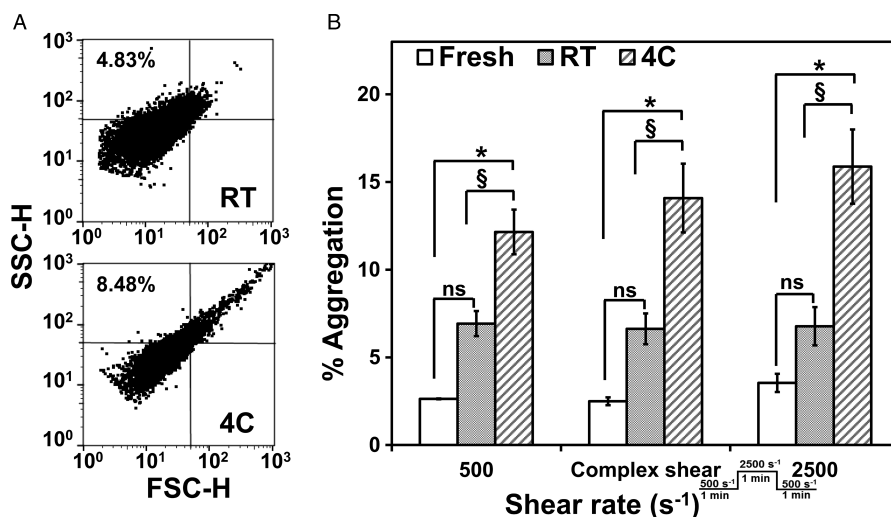
During dynamic mechanical analysis, the viscoelastic properties of the clot, namely, clot strength ( $G'$ , elastic moduli) and viscosity ( $G''$ , viscous moduli), steadily increase over time and attain a constant value (Fig. 3A). Events include a short lag phase because of the direct involvement of thrombin in converting fibrinogen to fibrin, followed by a log phase representative of rapid fibrin polymerization and terminating in constant

clot strength, an indication of gel stability. All samples demonstrated gel-like properties with  $G'$  greater than  $G''$ ,<sup>22,23</sup> which is typical of cross-linked fibrin (Fig. 3A). However, as shown in Figure 3B, the elastic moduli (clot strength) and viscous moduli (viscosity) of RT-stored platelets were threefold to fivefold lower than that of fresh platelets, but these values for fresh and 4°C-stored platelets were comparable with each other.

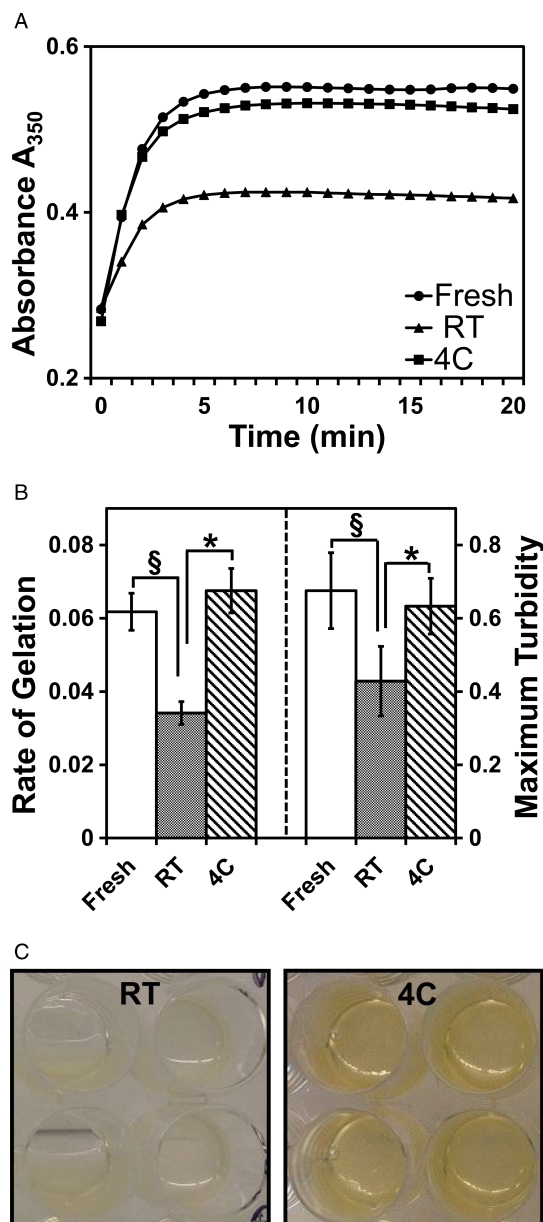
## DISCUSSION

In this work, we report that cold storage of platelets enhances shear-induced aggregation and improves clot strength at Day 5 compared with storage at RT. Establishing early hemorrhage control with hemostatic products after trauma is essential because emerging data suggest that such a strategy is associated with improved outcome.<sup>24–27</sup> Furthermore, current prehospital care of traumatic bleeding focuses on hypotensive resuscitation because of the risk of rebleeding.<sup>25,28–31</sup> A platelet product that forms stronger clots and is resistant to the disruption in the face of normalized pressure could be helpful in the prehospital setting, particularly for patients with spinal or brain injury. Our results demonstrate that cold-stored platelets form stronger clots, suggesting that they could result in more effective hemorrhage control.

Circulating platelets experience a range of shear stresses from low levels on the venous side (500 per second) to high on the arterial (2,500 per second). As they travel through changing vessel diameters, they are exposed to complex shear profiles with low- and high-shear segments.<sup>32</sup> While platelet aggregation in response to shear forces is well documented,<sup>33</sup> there is a paucity of data regarding shear stress effects on stored platelets.<sup>7</sup> Consistent with previous studies, we observed that short exposure to shear stresses causes fresh platelets to aggregate.<sup>19</sup> For stored platelets, the extent of shear-induced aggregation was dependent on storage temperature. While storage at



**Figure 1.** SIPA. Fresh or stored platelets were subjected to shear rates of either steady 500 per second or 2,500 per second for 3 minutes or a combination of 500 per second, 2,500 per second, and 500 per second for 1 minute each (complex) at 37°C, and platelet aggregation was assessed by flow cytometry. A, Aggregation was estimated from an increase in platelet population outside the single platelet gate compared with static controls. B, Platelets stored at 4°C aggregated significantly more than fresh or platelets stored at RT ( $n = 3$ , \* $p < 0.05$ ).



**Figure 2.** Clot turbidity analysis. Kinetics of gelation was monitored in fresh or stored platelets after the addition of 0.16-U thrombin and 3-mM calcium by measuring UV absorbance at 350 nm. **A**, Representative traces of the increase in turbidity during gelation. **B**, Rate of gelation estimated from the initial rate of absorbance, and clot turbidity is the absorbance attained at steady state ( $n = 4$ ,  $*p < 0.05$ ). **C**, Representative photographs of the clot formed in stored platelets.

RT did not have a significant effect, storage at 4°C enhanced aggregation compared with fresh platelets. We and others observed that, upon stimulation with physiologic agonists such as adenosine diphosphate or collagen, platelets stored at RT show a muted response compared with the enhancement seen in those stored at 4°C.<sup>15,34,35</sup> Thus, storage conditions affect platelet hemostatic function similarly, whether in response

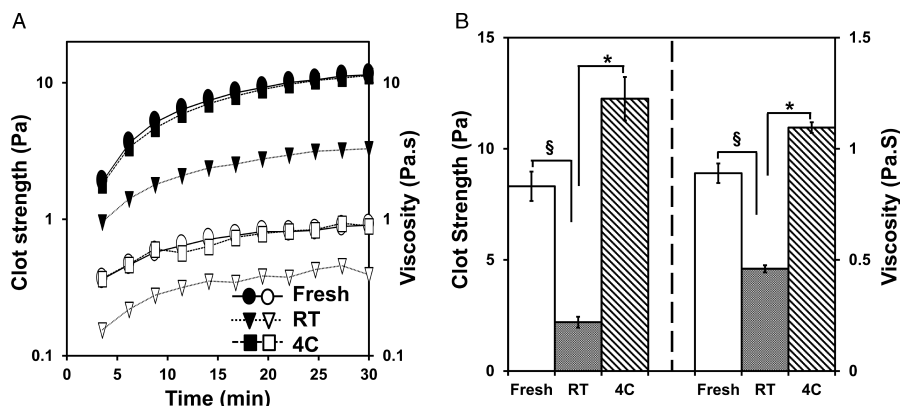
to mechanical, that is, shear stress, or chemical agonists. We previously reported that cold storage for 2 days increased SIPA in PRP,<sup>7</sup> and the present study demonstrates that similar effects are seen at Day 5. The heightened aggregation of 4°C-stored platelets under shear conditions may be attributable to two complementary mechanisms. Cold storage causes clustering of platelet surface GP Ib $\alpha$ , the mechanosensory receptor; thus, it may increase the avidity of GP Ib $\alpha$  for von Willebrand factor, its multivalent ligand, under shear conditions.<sup>7,33,36</sup> Second, because cold storage partially activates platelets, either as described earlier or via other mechanisms, and low temperature generally preserves the internal enzymatic machinery in cells, the platelets are thus “primed” for a maximal response to shear forces.<sup>34,37</sup> In contrast, shear forces and agonists elicit less aggregation on 5-day-old RT-stored platelets compared with fresh platelets, an effect that is attributable to aging and senescence during storage.<sup>35,38</sup>

Platelet activation and aggregation lead to clot formation, beginning with the linear polymerization of fibrin monomers, followed by fibrin cross-linking and gelation, which stabilizes the clot.<sup>20,21,38,39</sup> The rates of gelation between fresh and 4°C-stored samples were comparable, suggesting that the factors contributing to fibrin polymerization, namely, fibrinogen levels, plasma factors such as factor XIII or plasminogen activation inhibitors, and thrombin generation by platelets, are preserved during cold storage. Conversely, the significant drop in gelation rate in RT samples indicates a loss of one or more of these factors. The turbidity of the clot is linked to fiber diameter, with thick fibers producing opaque gels, and fiber thickness is strongly correlated with the clot strength.<sup>23,40,41</sup> The lower opacity of clots formed from RT-stored platelets compared with fresh and 4°C-stored suggests that RT storage compromises the ability to form thick fibers, but refrigeration does not.

Findings from clot strength analysis support this conclusion. We previously reported increased clot strength in 4°C-stored platelets;<sup>42</sup> however, the data were obtained via thromboelastography, a technique that is not designed to study clot rheology.<sup>43</sup> Clots are viscoelastic materials characterized by both elastic (solid) and viscous (liquid) properties and are best characterized under controlled strain/shear conditions using a rheometer. The elastic and viscous moduli of clots from fresh platelets measured in this work are in agreement with the published literature.<sup>22,42,44</sup> Refrigerated platelet clot strength, defined as the elastic modulus, was similar to fresh platelets, but corresponding values from RT-stored platelets were fivefold lower. These differences corroborate our recent observations on the effect of storage temperature on thromboelastography maximal amplitude.<sup>11</sup> The quantitative correlation between structure and rheology is not yet understood, but several factors are known to contribute to clot rheology, including fiber thickness, branch point density, fibrin concentration, and platelet contractility.<sup>23,39</sup> Taken together, our data indicate that platelet storage at 4°C, but not RT, generates clot structure and stiffness comparable with fresh platelets and allows us to speculate that 4°C may permit storage beyond 5 days.<sup>6</sup>

In summary, we have demonstrated that platelets stored at 4°C for up to 5 days are more responsive to aggregation stimuli and form stronger clots compared with platelets stored





**Figure 3.** Clot rheology. The viscoelastic properties of clots formed from fresh or stored platelets after the addition of 0.16-U thrombin and 3-mM calcium by dynamic mechanical analysis. *A*, Representative traces of clot strength or elastic moduli,  $G'$  (closed symbols), and viscosity or viscous moduli,  $G''$  (open symbols) during gelation. *B*, Elastic and viscous moduli of RT-stored platelets were significantly lower than 4°C-stored platelets ( $n = 4$ , \* $p < 0.05$ ).

at RT. These attributes suggest that 4°C-stored platelets may be a better transfusion product to treat acute bleeding, where immediate hemostasis rather than circulating lifespan is the priority, and preliminary clinical studies demonstrating the hemostatic efficacy of 4°C-stored platelets<sup>13,14,45</sup> corroborate the hypothesis. Furthermore, findings from this study indicate, but yet to be proven, that cold-stored platelets may also decrease incidence of recurrent hemorrhage due to the “popped clot” phenomenon. This could be particularly beneficial in patients with traumatic brain or spinal injury, who need to be maintained at higher blood pressures to support perfusion. Multicenter clinical studies of 4°C-stored platelets in acutely bleeding patients are indicated to determine whether refrigerated platelets will minimize time to hemostasis and rebleeding events.

#### AUTHORSHIP

P.M.N. designed and performed the experiments, analyzed the data, and wrote the first draft of the manuscript. H.F.P. designed the experiments and analyzed the data. A.P.C. designed the experiments, provided reagents, and analyzed the data. A.K.R. designed the experiments, provided reagents, analyzed the data, wrote the manuscript, and coordinated the progress of the project. All authors contributed to the final manuscript.

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#### DISCLOSURE

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